ANALYSIS OF THE FUNCTIONAL HETEROGENEITY WITHIN HUMAN T CELL SUBSETS UTILIZING THE MONOCLONAL ANTIBODY OKT10

Paul Bennet Rothman

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ANALYSIS OF THE FUNCTIONAL HETEROGENEITY WITHIN HUMAN T CELL SUBSETS UTILIZING THE MONOCLONAL ANTIBODY OKT10

A Thesis Submitted to the Yale University
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by

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ABSTRACT

Previous studies have attempted to correlate expression of certain surface antigens on human lymphocytes with functional properties of the cells. The T cell marker OKT4, which in initial studies was shown to identify T cells containing helper activity, has more recently been shown to also react with cells that have both suppressor and cytotoxic activity. The OKT8 reactive cells, which originally were thought to contain cytotoxic and suppressive cells, have been shown to also contain cells which enhance B cell differentiation. In order to further define these functionally heterogeneous populations, experiments were performed with OKT10, a monoclonal antibody reactive with an antigen present on all thymocytes and a small percentage of resting T lymphocytes, but expressed in varying proportions on activated T cells. Depletion of the OKT10 reactive T cell subset in normal resting T cells does not affect the ability of these cells to generate cytotoxic cells or to either enhance or suppress B cell differentiation. However, when unselected T lymphocytes were cultured for six (6)

days in mixed lymphocyte reaction and then depleted of OKT10 reactive cells, the ability of these cells to generate a cytotoxic response was eliminated. Also, depletion of the OKT10 reactive T cells from within the non-irradiated activated $T8^+$ cell population did not affect the ability of these cells to suppress B cell antibody production. Experiments directed at studying the heterogeneity within the irradiated activated $T8^+$ positive cells, which in previous studies have been shown to enhance B cell differentiation at sub and supra optimal levels of help but suppress B cell differentiation at optimal levels of help, revealed that the depletion of OKT10 reactive cells eliminated the suppression activity at optimal levels of help.

These experiments provide evidence that the OKT10 antibody can be used to isolate functionally distinct populations of the OKT8 reactive T lymphocyte subset.

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Introduction

Various types of cells participate in an immune response. The major groups are the lymphocytes, which until Gowans identified them as important cells in the immune system (17), were considered mainly uninteresting and non-functional. Lymphocytes are divided into two major categories, T and B lymphocytes. B cells, the progenitors of antibody-forming cells, derive from bone marrow stem cells, through a process of antigen-independent maturation that takes place in the bursa of Fabricius in birds, but whose location has not been defined in humans. The T lymphocytes responsible for regulating functions and cellular immunity derive in all species from bone marrow stem cells that go through a maturation stage in the thymus after which they are immune competent.

T lymphocytes are essential for the full expression of immunity due to their participation in various immune functions. T cells function as regulatory cells, modulating B cell differentiation and thereby affecting immunoglobulin production. This activity has been divided into helper function, whereby immunoglobulin (Ig) production increases or suppression function, where the ability of B cells to produce immunoglobulin is decreased. T cells are involved in cellular immunity reactions, which include reactions of delayed sensitivity, contact sensitivity, or resistance to certain infectious agents (i.e., viruses, intracellular bacteria). T cells are the major cells involved in transplantation immunity, which involves both rejection of allogeneic tissue and graft-verses-host reactions. Finally, T cells can act as cytotoxic cells whereby they

have the capacity to kill other cells. T cells have been thought to be able to generate these activities by their ability to recognize and interact with both antigens and autologous cells.

A major question arose as to whether this diversity of T cell functions reflected a functional heterogeneity of T cells existing prior to antigen stimulation. This was addressed by Cantor and Boyse working with mice (18, 19), who asked "...is it possible to separate subclasses of T cells from non-immune animals, that are already determined to express respectively, helper activity or cytotoxic activity before they encounter antigen?" (18)

Cantor and Boyse developed alloantisera that defined a panel of cell-surface differentiation components, called Ly antigens, which are expressed exclusively on the surface of thymocytes and T cells. They showed that unique combinations of these surface products are expressed by distinct subsets of T cells which have unique immunologic functions. Ly1 cells could be activated by I-region incompatabilities (i.e., differences in the "self" class II antigens) to generate helper activity (increased B cell differentiation). Ly2,3 cells accounted for virtually all T cell mediated cytotoxicity when stimulated by K/D differences (i.e., differences in "self" class I antigens) and were able to suppress the helper activity of Ly1 cells.

Jandinski, et al (20) extended these observations to T cells that were polyclonally activated by concanavlin A (Con A). After this non-specific activation, it was again shown that Ly2,3 cells suppressed antibody response and Ly1 cells enhanced "helped" antibody response. With further study it was found that an

antigen defined by the L3T4 monoclonal antibody better defined the T cell subset that was exclusive of Ly2,3 cells and had class II MHC restriction (99).

In studying T-cell ontogeny, it was found that the maturation from stem cell to thymocyte was associated with the acquisition of TL, Thy1, and Ly1,2,3 surface antigens. With further maturation a subset of thymocytes lose TL on their surface associated with a decrease in Thy1 antigen and an increase in H2 antigen. In addition, a significant number of cells either lose Ly1 or Ly2,3 on their surface, thereby forming $Ly2,3^{+}$ and $Ly1^{+}$ respectively. These peripheral subsets were noted to have the above functions. (31) Another population retained both the Ly1 and Ly2,3 antigens and form a subset of Ly1,2,3 cells in the periphery which serve to modulate the immune response by either differentiating into Ly1 helper cells or Ly2,3 suppressor cells (31).

Investigators tried to determine if the functional heterogeneity of human T cells could be defined by differences in cell surface characteristics. Strelkauskas, et al (22) used sera from children with severe juvenile rheumatoid arthritis which contained antibodies reactive with 30% of peripheral human T cells in an attempt to separate T cells subsets. These autoantibodies were able to separate out a subclass of human T cells which was able to enhance B cell immunoglobulin production. (22)

Moretta, et al (23) used the ability of T cells to bind different immunoglobulin classes to distinguish T cells with different functions. T cells with helper function could be localized in a subset of T lymphocytes bearing surface receptors for Fc portion

of IgM (T_M^+) while in contrast, suppressor activity was associated with a T cell subset which had a receptor for the Fc portion of IgG (T_G^+ cells).

Reinherz, et al then developed two rabbit anti-human T lymphocyte heteroantiseras to define functional human T cell subsets. (14, 24-26). One heterologous anti-human T cell serum, TH_1, reacted with 50% of peripheral T cells. Depletion of cells reactive with this antisera abolished the response in MLC and elaboration of mitogenic factor, but did not affect the proliferative response to soluble antigens. (14). They found that TH_2, a second heteroantisera, reacted with 20% of human peripheral T cells and this subset contained both the cytotoxic effector and Con A-inducible suppressor cell population. In contrast, cells that did not react with TH_2 provided helper function in a variety of systems and could modulate the generation of Con A inducible suppressor cells. (25)

The above studies, utilizing heteroantisera, spontaneous auto antibodies, and the ability to bind the Fc of immunoglobulins demonstrated that human T cells, which appeared relatively homogenous morphologically, consisted of subclasses of cells with differing functions that could be fractionated by differences in cell surface antigens. These techniques had limited clinical value due to the disparity of antibody preparations obtained, frequent low titers, and the multiple absorptions required for production of specific heteroantiserums.

The hybridoma technique of Kohler and Milstein (27) allowed production of antibodies specific for a single antigen. The

technique involves immunizing BALB/CJ or CAF mice with human T cells, then harvesting the mice spleen and fusing these cells with myeloma cells. The fusion cells are selected in HAT (hypoxanthine-aminopterin-thymidine) supplemented medium and antibody producing hybrids were identified by a fluorescent labelled goat anti-mouse antibody. A single clone of cells was then injected back into a mouse and a malignant ascites (containing monoclonal antibody) was harvested. Presently there are two series of monoclonal antibodies that have been extensively studied. The Leu series developed by Evans, et al (11) and the OKT series produced by G Goldstein and P.C. Kung. (7, 32).

Reinherz and Schlossman have used the OKT series to study T cell maturation. In parallel to the murine work of Cantor and Boyse, they have shown that changes in cell surface antigens mark various states of human T cell ontogeny (28, 29). In human bone marrow, TdT$^+$ lymphoid precursors have shown to react with OKT10, but no other T cell antigens (30). In the thymus virtually all thymocytes bear OKT 10, though major differences in other cell surface antigens are found on thymocytes in different stages of maturation. It appears 10% of thymocytes bear only OKT10, while with further maturation they acquire a thymocyte distinctive antigen, OKT6, and concurrently express OKT4, OKT5, and OKT8. Cells with the above phenotype comprise 70% of thymocytes. With further maturation cells lose OKT 6 on their surface, but acquire OKT3 and OKT11. These cells also segregate into mutually exclusive subsets which display either OKT4 or OKT5/8 on their surfaces. This would seem to parallel the

distribution of the L3T4 and Ly2,3 antigens in the mouse system. Finally, the thymocytes that are exported into the periphery lose OKT10 on their surface. The above scheme of maturation is summarized in figure 1. Thus, peripheral lymphocytes are subdivided into two mutually exclusive groups, one bearing OKT3, OKT11, and OKT4 antigens, and the other bearing OKT3, OKT11, OKT5 and OKT8 cell surface molecules.

Given the existence of two distinct subpopulation of T cells, studies were undertaken to see if evidence could be found, as it had been in the mouse system, correlating cell surface markers with distinct T cell function.

Initial studies by Reinherz & Schlossman found that OKT4 reacted with the same T cells which had not reacted with TH_2 heteroantisera (TH_2^- cells). These T cells represented 60% of peripheral blood lymphocytes (PBL). Functionally, the OKT4 reactive cells ($T4^+$) contained the entire proliferative capacity of the T cell population to soluble antigen. These cells also responded to alloantigens and mitogens such as Con A and PHA. The $T4^+$ subset developed a small amount of cytotoxic activity after activation, and were able to greatly increase the amount of cytotoxicity developed by $T4^-$ cells when combined prior to sensitization. Importantly, $T4^+$ positive cells were shown to greatly enhance B cell differentiation in both pokeweed mitogen (PWM) driven and antigen-stimulated system (33,34).

The ability of $T4^+$ cells to increase B cell immunoglobulin (Ig) productions and to increase proliferation of all lymphocyte subclasses was shown to be mediated by non-specific soluble "helper

factor(s)" (35). Thus, the $T4^+$ subset of T cells in humans performed many of the same immune functions that Ly1 cells performed in mice.

T cells that did not react with OKT4 were found to react with OKT5 and OKT8. These antibodies reacted with 30-40% of peripheral T cells, all of which also reacted with TH_2 heteroantisera. (36). OKT5 and OKT8 antibodies were found to react with different molecules, both of which were present on the same subset of T cells (37). The cells ($T8^+$) were virtually unresponsive to soluble antigen, but proliferated well to the mitogen, Con A, and to alloantigens. (36). After activation by Con A, $T8^+$ cells were able to suppress autologous T cell proliferative responses to alloantigens. This same $T8^+$ subset also suppressed B cell immunoglobulin production (33). Moreover, the effector function for cell-mediated lympholysis was found to reside in the $T8^+$ subset. (33,34,37). Thus, the human T cells reactive with OKT5 and OKT8 ($T8^+$) were functionally similar to the Ly2,3 cells in the murine system.

Reinherz & Schlossman studied the correlation between the reactivity of OKT antibodies and that of prior methods of T cell fractionalization. As stated above, TH_2 reactive cells were found to react with OKT5 and OKT8, while cells that didn't react with TH_2 did react with OKT4. Studies that investigated the OKT antibodies in relation to receptors for the Fc portion of immunoglobulins were difficult to interpret due to the instability of the T_M and T_G phenotype. (39).

Concurrently with the work of Reinherz & Schlossman, Evans et al at Sloan-Kettering developed the Leu series of monoclonal antibodies. They produced Leu 2 (T8), an antibody that reacted with the same T cells as TH_2, and Leu 3 (T4), which reacted with cells unreactive with TH_2. In a parallel fashion, Gatenby & Engleman found the Leu 2 reactive subset of T cells was responsible for cytotoxic/suppressor function while the Leu 3 cells were involved in helper/induced activity. (11,40).

Utilizing the OKT series of monoclonal antibodies, along with gamma radiation, Y. Thomas and L. Chess further investigated the interactions of lymphocytes responsible for immune functioning. They found that in a PWM driven B cell differentiation assay, the helper function of $T4^+$ cells was within a radiosensitive subset of T cells. They also found that $T8^+$ suppressor activity was generated by a radiosensitive cell. Further, the suppression of B cell differentiation mediated by $T8^+$ cells required the presence of a radiosensitive $T4^+$ cell subset. (8). These findings were extended to T cells reactive to alloantigens, so that a radiosensitive subset of $T8^+$ cells was shown to suppress $T4^+$ response to stimulation by alloantigens. (41). Thomas and Chess were able to isolate supernatant, derived from $T4^+$ cells, that enhanced B cell differentiation. Production of this "helper factor(s)" could be suppressed by $T8^+$ cells, and the authors postulated this could be the mechanism by which $T8^+$ cells mediated their activity on B cell differentiation. (41).

During these studies it was noted that addition of greater numbers of $T4^+$ cells to B cells did not result in a linear increase

in B cell differentiation (as measured by immunoglobulin producing cells), but instead a decrease was noted after an initial zenith. Thomas and Chess went on to show that addition of $T4^+$ cells, which had been activated in vitro by pokeweed mitogen (PWM), could suppress the helper effect of fresh $T4^+$ cells in B cell differentiation. They showed that this was not secondary to the emergence of $T8^+$ cells, but was mediated by a subset of radiosensitive $T4^+$ cells. (13).

The existence of $T4^+$ suppressor cells was further defined by use of OKT 17 which was found to react with activated $T4^+$ suppressor cells. (42). The ability of $T4^+$ cells to suppress has since been shown with human cord blood (43), adult T cell leukemia cells (44), and most recently with alloreactive T cell clones (45). Thus, $T4^+$ cells were shown to be capable of both suppressor and helper function in relationship to B cell differentiation.

Other studies were undertaken to identify further heterogeneity within the $T4^+$ cells subset. Utilizing T cell clones, several investigators have shown that cytotoxic T cells can bear the OKT4 cell surface marker. (46,47). Thus experimental evidence from several different groups have indicated that $T4^+$ human T cell subset has the ability to perform a diverse range of immune functions.

The OKT8 reactive cells have been shown by Reinherz & Schlossman to be responsible for the majority of cell mediated lympholysis (CML) and to be able to exert suppressor function on B cell differentiation. Recently, Thomas & Chess utilized PWM

activation and irradiation to further investigate the immunoregulatory effect of $T8^+$ cells. They found that activated non-irradiated $T8^+$ cells suppressed $T4^+$ mediated B cell Ig production at all levels of Ig production. However, activated irradiated $T8^+$ cells showed suppressor function only at optimal levels of Ig production. At supra or sub-optimal levels of $T4^+$ mediated B cell differentiation, addition of activated irradiated $T8^+$ cells enhanced Ig production (3). Thus, the $T8^+$ T cell subset has also been shown to be heterogenious, containing cells capable of expressing 1) suppressor 2) cytotoxicity and, after activation 3) amplifier activity in different systems.

Similar findings of heterogeneity in what were thought to be well-defined T cell subsets have also been found in the murine system. (48-50). Both Ly1 and Ly2,3 cells have been shown to appear on cytolytic T cell clones. (48,50). Subclones of a single cell clone have been shown to perform proliferative response, cytolytic activity, and allohelp reaction with B cells. (48).

Gershon, et al (49) used an antisera directed at a polymorphic gene product(s) controlled by the I-J subregion of the major histocompatibility complex (MHC) to help define the disparity in the murine T cell system. Ly1 cells reactive with the anti-I-J serum ($I\text{-}J^+$) were selective for their ability to induce suppressive activity in the Ly2,3 cells, while Ly1 cells not reactive with anti-I-J serum ($I\text{-}J^-$) were specialized in their ability to induce B cell differentiation. (51).

Gershon used the I-J antisera to also look at Ly2,3 cell function. Ly2 T cells not reactive with I-J antisera ($I\text{-}J^-$) were

the effector cells that suppress Ly1 helper T cell activity. The Ly2, I-J$^+$ subset contained cells that interact with Ly1,2 T cells to inhibit Ly2 mediated suppressor cell activity. This activity was termed contrasuppression and was thought to consist of a regulatory loop controlling the level of suppression generated in an immune response which involved a Ly2 I-J$^+$ cell that could induce an Ly1 cell to "contrasuppress" Ly2 mediated suppression. This inducement of contrasuppression was mediated by a soluble factor. Thus, Gershon hypothesized four populations in the Ly2 T cell subset with distinctive functions: 1) killer; 2) suppressor; 3) cells that amplify suppressor activity; and 4) cells that induce contrasuppression. (49).

Another approach towards investigating T cell subsets, as defined by cell surface antigens, involved attempts to block functions of known T cell subsets utilizing the antibodies directed to the surface antigens that defined these subsets. This approach addressed the issue of whether these molecules were involved in the functions exhibited by the T cells they appeared on.

In the murine system, early studies investigated the effect of monoclonal antibodies directed towards the Ly2,3 molecular complex, in the absence of complement, on the cytolytic function of these cells. Disappointingly, some studies showed the antibodies could block the cytolytic activity (52,54) while others were unable to show an effect (53,55). Using clones of cytotoxic T cells, it was shown that there was heterogeneity within subclones in their ability to be effected by anti Ly2,3 antibodies. (56).

Similar experiments have been performed with OKT antibodies to human T cells. Early experiments studying the effect of OKT4 without complement, on T cell function revealed that the antibody did not block proliferation to alloantigens, generation of cytotoxic T cells, or the effector cells of cytotoxicity (57). To further evaluate the role the molecule might be playing in T cell function, several monoclonal antibodies were produced to different epitopes of the OKT4 molecule. The OKT4A and OKT4B monoclonal antibodies were shown to prevent cytotoxic activity of $T4^+$ CTL clones and this effect could be overcome by lectin binding. (58,63). Other monoclonal antibodies to the OKT4 molecule, OKT4C and OKT4D, were unable to prevent the cytotoxic activity of $T4^+$ CTL clones. (63).

The ability of this battery of antibodies to affect B cell differentiation enhancement by $T4^+$ cells was investigated by L. Chess, et al. OKT4A and OKT4E were found to inhibit the induction of B cell differentiation, while OKT4, OKT4B, OKT4C, and OKT4D had either no effect or inconsistent effects. (4,65). The above studies indicated that the OKT4 molecule was important in cellular recognition events.

Studies were also conducted with the OKT8 antibody to study its effect on T cell function in the absence of complement. It was shown that OKT8, placed with CTL at the effector phase, diminished the cytotoxicity by 20-30% (59,60). The antibody could totally inhibit the cytotoxicity generated by $T8^+$ clones CTL, and this effect was also overcome with lectin binding. (58).

To study the T8 molecules function in suppression of B cell differentiation, several monoclonal antibodies to different epitopes of OKT8 molecule were produced and tested for their effect on $T8^+$ suppression of $T4^+$ induced B cell differentiation. OKT8E and OKT8G were found to inhibit suppressor activities in the absence of complement, while OKT8B, OKT8C, OKT8D, OKT8F, and OKT8H did not affect this function (65). Thus, the OKT8 molecule was also felt to be involved in T cell antigen recognition. (58).

Several groups have also studied the effect of OKT3, which reacts with 95% of peripheral T cells. In a group of experiments it was shown that placing the antibody with unselected T cells, without complement, prevented proliferation to soluble antigens and to cell surface alloantigens (57-59). OKT3 was also shown to prevent generation of cytotoxic T cells (57,60) and to prevent CML at the effector phase (58-60). This inhibition of CML could be overcome by culturing the CTL and target cells with lectin (58). These results indicated that OKT3 was not necessary for the physical act of cytotoxicity (58). Later studies revealed that OKT3 did not block cytotoxicity at a target-binding step, but inhibited a subsequent lytic step. (15). OKT3 was also shown to be mitogenic (61,60) and to increase production of γ interferon by T cells (61). Further studies indicated that binding OKT3 modulated the cell surface of T cells, resulting in shedding of the OKT3 molecule and resultant loss of the above functions. Reinherz, et al were able to correlate the reappearance of OKT3 on the surface of T cells with the reacquisition of T cell functions. (62).

Though early studies in both the murine and human systems showed that T cell subsets (isolated by depletion studies utilizing monoclonal antibodies and complement) had some general functional characteristics, later studies revealed the specificity of these correlations seemed less assured. Experiments exploring the function of the molecules themselves indicated that several were probably involved in T cell function. To further evaluate the physiologic role of different T cells , monoclonal antibodies to different activation antigens were studied. Activation antigens are those cell surface molecules that are expressed in a greater frequency on cells that are activated.

Feeney and Hammerling produced antisera to Ala-1, a murine alloantigen which was expressed only on activated peripheral murine B and T cells (66). Using depletion studies they noted that anti-Ala-1 was reactive with cytotoxic T cells, helper T cells, and IgM and IgG plaque forming colonies (B cells). (67).

Kimura and Wigzell produced an alloantisera to a murine cell surface glycoprotein T145 which was absent on resting T lymphocytes but was found on killer T cells. Depletion of activated cells by I-J and complement did not eliminate the T145 reactive cells, which the authors concluded was probable evidence that T145 was not present on suppressor cells (68).

In the human system several different activation antigens have been identified by monoclonal antibodies. OKT9 reacts with thymocytes and some human T-ALL (T cell leukemia) cells, but not with normal T or B cells (29). In T cells, after activation by PHA, OKT9 reactivity was detectable in three (3) days. (69).

Greaves et al showed that OKT9 antibody bound to the transferrin receptor on human cells (69) and thereby showed that transferrin receptors were expressed on the surface of leukemic cells.

Waldmann, et al produced a monoclonal antibody termed Anti-Tac which did not react with fresh peripheral lymphocytes (PBL), but appeared on T cells activated by mitogen or allogeneic cells (70). They showed that Anti-Tac reacted with activated T cells mediating suppressor function, radioresistant helper function, and cytotoxic killer T cells. Cells unreactive with Anti-Tac contained helper cells. (71). Further study revealed that Anti-Tac reacted with the human membrane receptor for T cell growth factor (TCGF or interleukin-2). (72).

The best studied activation antigen to date in the human system is that designated Ia, which is the product of the I region of the MHC in humans. Schlossman, et al developed a monoclonal antibody to Ia antigen which was reactive with B cells, monocytes, a sub population of null cells and leukemic blast cells, but was minimally reactive with peripheral T cells. (74). Upon activation by alloantigen Ia appeared on cytotoxic T cells. Depletion of the small number of Ia^+ cells on fresh T cells did not eliminate the appearance following activation of cytotoxic cells bearing Ia. (73). Further investigations utilizing Ia indicated that cells activated with mitogen or tetanus toxoid expressed Ia on the inducer T cell population exclusively. (74). Studies using cloned cell lines revealed that cytotoxic T cells were detectable in both Ia reactive and non-reactive populations. (75). Experiments probing the ability of antibodies to affect cytotoxicity found that addition of Ia

antibody without complement had no effect on the generation of T cell mediated cytotoxicity. (57, 59).

Several investigators have noted an elevated level of Ia positive T cells in various disease states. Among these are presumed auto-immune diseases such as Grave's disease (76), rheumatoid arthritis and systemic lupus erythematosus (77), type I diabetes mellitus (78), and graft vs. host reactions. (79). Elevated levels are also seen in several infectious diseases such as infectious mononucleosis (80), and acute bacterial infections. (77).

One monoclonal antibody OKT10, which has previously been described in respect to its reactivity with human thymocytes has also been shown to react with an activation antigen (81). This molecule has been studied by several different investigators for its specificity to different stages of leukocyte differentiation.

OKT10 was isolated by immunization of human thymocytes into BALB/CJ mice. The resultant monoclonal antibody was shown to react with greater than 95% of thymocytes. It was noted that the OKT10 was reactive with 5-10% of peripheral T cells and approximately 15% of peripheral E^- cells. (7). Reinherz, et al used the specificity of OKT10 for thymocytes to describe the cell surfaces changes during maturation of thymocytes. (28,29).

While exploring the reactivity of bone marrow cells with the OKT battery of monoclonal antibodies, it was discovered that unlike the other antibodies, OKT10 reacted with bone marrow terminal transferase positive (TdT^+) cells. (30,82). It was also found that the strongly $OKT10^+$ cells composed 18% of the bone marrow population. These cells included not only TdT^+ cells, but

they were almost all Ia^+, and some were bone marrow B lymphocytes. (30,82). Further, Janossy, et al, showed that 10% of bone marrow cells were weakly staining $OKT10^+$ large cells and had promyelocyte and myelocyte morphology. Mature myeloid cells and erythroid precursors (normoblasts) did not react with OKT10. (30). Further evidence that OKT10 was reactive with early hematopoietic stem cells comes from its reactivity with some non-T-ALL, AML, as well as the majority T-ALL cells. (83-84).

As mentioned, OKT10 reacts with 5% of peripheral T cells and 15% of peripheral E^- cells. Investigators showed that within the E^- cells, OKT10 reacted with 5-10% of peripheral B cells (28). Ortaldo, et al, studied natural killer (NK) cells and reported 60% of purified NK cells were reactive with OKT10 and that depletion of OKT10 reactive cells within this population eliminated lytic activity. Further, though 20% of NK cells were reactive with OKT8, depletion of $T8^+$ cells did not eliminate lytic activity (85). Thus, OKT10 reacted with the effectors of NK cytoxicity.

Because of its distribution on a variety of leukocytes, investigators characterized the molecule which reacted with OKT10. It was noted that the molecule was a glycoprotein of molecular weight 45-46K associated with a smaller peptide of 12K, with a mobility on gel electrophoretic studies similar to that of B2-microglobulin (B_2m). (86). Interestingly, HLA antigens in man are of similar size and are associated with B_2m. Further study using immunoprecipitation revealed that this smaller peptide attached to OKT10 was distinct from B_2m. (86,81).

Under non-reducing conditions the OKT10 antigen was found to have a molecular weight of 37K. This difference was thought to indicate the probable presence of stabilizing intrachain sulfhydryl bridges within the molecule. (81). Due to its ability to form protein micelles and to require detergent for its isolation, it was felt that the glycoprotein may contain hydrophobic regions and that it may be an integral membrane protein. (81).

Further information on the relationship between OKT10 antigen and its membrane properties came from pertubation of the cell surface of intact cells. Greaves, et al, attempted to induce leukemic cell lines to differentiate in vitro by TPA, and then study changes in cell surface markers. They found that in these cells, with reduction in TdT reactivity (increasing differentiation) there was also a decrease reactivity with OKT10. (83).

Goldstein, et al studied T cell surface changes after modulation with OKT3 with and without cross-linking of the bound OKT3 antibody by a horse-anti-mouse antibody. They noted that both Ia and OKT10 reactivity greatly increased after cell perturbation triggered by the cross-linking of OKT3 antibodies. (87).

The above findings that pertubation of T cell membranes could change the reactivity of the cells with OKT10 antibody are interesting in light of Schlossman's report that upon activation with Con A, the reactivity of peripheral T cells with OKT10 antibody increased from 5% to 50%. (81).

The in vivo relevance of this phenomenon comes from studies of several pathologic states. Phenotypic studies of T cells in EBV-induced infectious mononucleosis revealed that acutely greater

than 50% of peripheral T cells react with OKT10, and this reactivity decreases with the resolution of the disease. (88). Similarly some AIDS patients have lymphocytes with a high percentage of OKT10 reactivity (Chess unpublished).

The ability to study OKT10 reactive cells has been limited due to the inability of the antibody (IgG_1) to bind complement. Recently, G. Goldstein has produced a new battery of monoclonal antibodies reactive with activation antigens. One of these, OKT20, was found to be an IgM (complement-binding) antibody that, by immunoprecipitation studies, reacts with different epitopes of the same molecule as OKT10 (G. Goldstein, et al unpublished). This monoclonal antibody renamed OKT10A was utilized in this study to investigate the role of OKT10 reactive cells in functional T cell studies in an attempt to further dissect the heterogeneity found in peripheral T cell subsets.

Materials and Methods

I. Lymphocyte Preparation and Isolation of Human T and B Cells:

Fresh peripheral blood lymphocytes (PBL) were isolated from consenting healthy human volunteers by utilizing the Ficoll-Hypaque density gradient centrifugation of Boyum (1). Lymphocyte sub-populations were then isolated by one of several different methods.

Highly enriched populations of T and B cells were isolated by methods previously described (2,3). PBL were washed in minimum essential medium (MEM) (Grand Island Biological Co., Grand Island, N.Y.) containing 5% fetal calf serum (FCS) (Microbiological Assoc., Bethesda, Md.) and then separated into surface Ig^+ (SIg^+) and surface Ig^- (SIg^-) populations using Sephadex G200 rabbit anti-human F $(ab)_2$ columns in the presence of 2.5mM EDTA. The Sig^+ populations were eluted with soluble Ig and subsequently further purified by complement (c) mediated lysis of residual T cells using OKT3 monoclonal antibody (5), thereby yielding purified B cells. The SIg^- population was further fractionated into highly purified T cell populations by the formation of E rosettes with sheep erythrocytes. (See, below).

An alternative method was also utilized to isolate enriched T and B cells. PBL were fractionated by their ability to form E rosettes with sheep erythrocytes (SRBC; Colorado Serum, Denver Co.). Cells were placed at a concentration of 20×10^6 cells per ml and were mixed with 5% SRBC and placed at 4^oc overnight. Subsequent separation into E^+ and E^- populations was achieved via

Ficoll-Hypaque centrifugation. E^+ cells were freed of SRBC by incubation with 0.83% ammonium chloride, and were washed in media three times yielding unselected T cells. E^- cells were either used as stimulator populations or further purified into B cells by C-mediated lysis of residual T cells and macrophages using OKM1, OKT3 and OKT11A monoclonal antibodies (4). (See, below).

Functional evidence for B cell purity was obtained by lack of activation of these B cells by pokeweed mitogen (PWM) in the absence of added T cells.

II. Characterization of the Monoclonal Antibodies, OKT3, OKT4, OKT8, OKT10, OKT10A, OKT11, OKM1, OKB1:

The monoclonal antibodies, OKT3, OKT4, OKT8, OKT10, OKT10A, OKT11A, OKM1, OKB1, were a gift of Drs. Patrick Kung and Gideon Goldstein, Ortho Pharmaceutical Corporation. The production and functional characterization have been previously described (58) except OKT10A and OKB1. The antibodies OKT3, OKT4, OKT8, and OKT11A all bind complement and are specific for human T cells. OKT3 is an IgG_2 which reacts with 90% of peripheral E^+ cells, OKT11A reacts with 100% of E^+ cells. OKT4 and OKT8 are both IgG_2 antibodies and react with mutually exclusive population of peripheral E^+ cells ($OKT4^+$ reacts with 50-60% of peripheral E^+ cells while OKT8 reacts with 30-40%). OKM1 reacts with approximately 80% of PBL adherent cells and about 20% of non-adherent cells. OKB1 is an IgG specific for peripheral B cells (G. Goldstein, unpublished), and was utilized as a control in several experiments involving T cell function. OKT10 is an IgG_1 (non-complement binding) which reacts with all

thymocytes, and a small number of peripheral T cells, B cells, and macrophages. OKT10A is a new IgM (complement fixing) monoclonal antibody which reacts with the same array of cells as OKT10, and by immunoprecipitation studies (G. Goldstein unpublished), has been shown to react to the same molecule as OKT10.

III. Isolation of Lymphocytes Subsets by Complement (c) Mediated Lysis Utilizing Monoclonal Antibodies:

The use of monoclonal antibodies and complement to isolate a subset of lymphocytes has been previously described (8). In experiments to isolate T cell populations $50x10^6$ unfractionated T cells were resuspended in 1 ml of ascitic antibody diluted 1/250 in final media (except OKT10A which was diluted 1/100) and incubated for one (1) hour at room temperature. Fresh rabbit complement was added at a final dilution of 1:12 and the cells were incubated at 37°C for one hour. The cells were then washed three times in MEM wash and counted. Analysis of the resulting populations showed cells treated by OKT4 and complement contained greater than 90% $OKT3^+$ cells, greater than 90% $OKT8^+$ cells, and less than 2% $OKT4^+$ cells, whereas the OKT8 treated cells contained greater than 90% of $OKT3^+$ cells, greater than 90% $OKT4^+$ cells, and less than 2% $OKT8^+$ cells. We use the notation $T4^+$ to identify cells treated with OKT8 and complement and $T8^+$ to identify cells after treatment with OKT4 and complement.

E^+ cells treated by OKT10A and complement are identified as E^+10^- cells. To eliminate T cells reactive with OKT10A in the $OKT4^+$ and $OKT8^+$ populations, a second complement mediated lysis

was performed, and these cells are identified as $T4^{+}10^{-}$ or $T8^{+}10^{-}$ respectively.

IV. Functional Studies:

A. Helper Function: - First Culture PWM Activation:

Isolated E^{+} cells were activated with PWM (Grand Island - Biological Co.) (10ug/ml) at a concentration of $2x10^{6}$ cells/ml in 25 cm^{2} surface area tissue culture flasks (Falcon, Oxnard, CA) for 72-96 hours at 37°C in a humid atmosphere containing 5% CO_2. Cells were then resuspended and washed extensively. Viability was then checked and the cells were fractionated into $OKT4^{+}$ and $OKT4^{+}10^{-}$ subsets by complement mediated lysis (see below). Each subset was then split and half of each was irradiated with 1,250 rads by using the Model M 38-1 gammator emitter (Isomedix, Parsippany, N.J.). Subsequently cells were added in graded numbers to appropriate secondary cultures.

Second Culture (ASSAY):

The ability of the PWM activated $T4^{+}$ and $T4^{+}10^{-}$ cells (first culture) to exert helper activity was determined by adding graded numbers of these cells to $1x10^{6}$ fresh autologous B cells. After five (5) days of in vitro sensitization in the presence of 10ug PWM, the cultures were harvested and assayed for plaque forming cells (PFC) activity using the reverse plaque assay (See, below).

B. Suppressive Function: - First Culture PWM Activated:

As above isolated E^{+} cells were activated with PWM for 72-96 hours, whereupon cells were washed and fractionated into OKT8 and $OKT8^{+}10^{-}$ subsets by C-mediated lysis (see below). In some experiments, some of these cells were separated and irradiated (as

above). In experiments studying irradiated $T8^+$ cell function the first culture PWM activation was for six (6) days in order to maximally increase OKT10 reactivity.

Second Culture (ASSAY):

The ability of PWM activated $T8^+$ and $T8^+10^-$ cells (first culture) to exert suppressor activity was assessed by adding graded numbers of these cells to $1x10^6$ fresh autologous B cells and $0.05x10^6$ fresh autologous $T4^+$ cells. After five (5) days of sensitization in the presence of 10ug PWM, cultures were harvested and assayed for PFC activity.

C. Reverse Hemolytic Plaque Assay for the Enumeration of Antibody-Secreting Cells:

The assay for the measurement of total Ig producing hemolytic plaque forming cells (PFC) was described elsewhere (9,10). Briefly, on the day of assay, cells were thoroughly washed in RPMI 1640 medium and resuspended. 50-100 ul aliquots were added to 0.9 ml of 0.5% liquid agarose (Seakem Agarose, Marine Colloids, Rockland, ME) containing 100 ul of an 11% suspension of srbc coupled by the chromic chloride method (98) to rabbit-anti-human immunoglobulin. This mixture was layered on a 60 x 15 mm petri dish previously coated with 5 ml of 0.5% liquid agarose and allowed to gel. The dishes were incubated for one hour at 37°C, in a humid atmosphere containing 5% CO_2, 95% air. One ml of a 1/100 dilution of rabbit anti-human IgG antisera was then added for an additional hour of incubation. Finally, the antisera was removed and 1 ml of a 1/10 dilution of absorbed guinea pig complement (Cedarlane Laboratories, Hicksville, NY)

was added for an additional hour. Plaques were enumerated in duplicate and the results expressed as the mean PFC/10^6 B cells in original culture. The standard error of the mean was always less than 20%. In addition, cell counts and viability (by dye exclusion) were performed on all cell cultures at the time of assay.

D. In Vitro Sensitization:

In vitro sensitizations were performed in sterile Linbro MR-2 microplates (Linbro Scientific Co., New Haven, Ct) as previously described (11). Briefly, each microwell contained $2x10^5$ responder T cells and $2x10^5$ irradiated allogeneic E^- stimulators suspended in 0.2 ml of final medium. In experiments examining effects of antibodies on sensitization, Ascites, OKT8, OKT10, or OKB1 antibodies were added to final medium (1/100 final dilution). Microtiter plates were incubated at 37°C in a 5% CO_2 95% air humid atmosphere for 6 days.

E. Proliferative Studies:

Cells were sensitized as above in flat bottomed microtiter plates. All experimental groups were assayed in triplicate. After six (6) days cultures were pulsed for 8-15 hours with 0.2 uCi of ^3H thymidine, specific activity 1.9 Ci/mM (New England Nuclear, Boston MA), harvested in the MASH II apparatus, and incorporation of ^3H thymidine was measured by liquid scintillation counting. Data are expressed as counts per minutes ± SEM (12).

F. Cell-Mediated Lympholysis:

Experimental T cells were stimulated as above by irradiated allogeneic E^- cells. These experimental T cells were either

unselected E^+ cells or in some experiments E^+ cells depleted of OKT10 reactive cells by C mediated lysis. Effector cells were harvested after six (6) days, washed twice and then either used in ^{51}Cr release assay, or depleted of OKT10 reactive cells by antibody and C treatment and then used in the assay. Allogenic as well as autologous target cells were cultured in final medium and stimulated by PHA (GIBCO) at 10ug/ml for the last four (4) days of the incubation. Before the assay $1x10^6$ target cells were pelleted and resuspended in 0.3ml of final media and labelled with 25uCi of sodium (^{51}Cr) chromate (specific activity 200-500 mCi/mg of Cr, New England Nuclear) for 1 hour. The targets were then washed three times and resuspended at $5x10^4$ cells/ml. Graded numbers of effector cells were then mixed with $5x10^3$ target cells in round-bottom microtiter plates and centrifuged 500 RPMx5 minutes and incubated for 5 hours at 37°C in a humid incubator with %5 CO_2. Each sample was done in triplicate wells. In some experiments antibodies were added to effector cells for 1 hour prior to addition of target cells (final dilution 1/100). After incubation, the cells were centrifuged 1,000 RPMx10 minutes and 100 ul samples of supernatant were removed and radioactivities determined in a gamma counter. Percent cytotoxicity was determined by ^{51}Cr release as follows:

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental release-spontaneous release}}{\text{Maximal release-spontaneous release}} \times 100$$

Maximal release was determined by treating $5x10^3$ of target cells with 100ul of 5% Triton X-100 for 5 hours at 37°C. Spontaneous release was determined by incubating target cells in medium

alone for 5 hours at 37°C. Non-Specific killing was determined by ^{51}Cr release from autologous targets and this value was subtracted from total cytotoxicity to determine specific cytotoxicity.

V. Cytofluorographic Analysis:

Phenotypic analysis of all cell populations was performed by indirect immunofluorescence using the monoclonal OKT antibodies and a fluorescein-conjugated goat anti-mouse Ig (G/M FITC) (Meloy Laboratories, Springfield, VA) utilizing a Model 30-H Cytofluorograf (Ortho Instrument, Westwood, MA). In brief, $5x10^5$ cells were treated either with OKT3, OKT4, or OKT8 at 1:10,000 final dilution, or OKT10 at 1:2,000 final dilution and incubated at 4°C for 30 minutes. After washing, 0.1 ml of a 1:40 dilution G/M FITC was added to the cell pellet (1:80 final dilution), mixed well, and incubated at 4°C for 30 minutes. Following this, the cells were washed two times and resuspended in 1 ml of PBS with 0.1% sodium azide. Mouse ascites fluid was included as a negative control. The cells were analyzed on the Cytofluorograf; the intensity of fluorescence per cell was recorded on the Cytofluorograf; the intensity of fluorescence per cell was recorded on a pulse height analyzer and those cells with fluorescence intensity greater than that of the ascites control were considered positive.

VI. Media:

WASH-Minimum essential medium (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 1% penicillin-streptomycin, 6mM Hepes buffer, 5% heat-inactivated FCS, and 0.05% sodium bicarbonate (Microbiological Associates).

Final-RPMI-1640 (Grand Island Biological Co.) supplemented with 1% penicillin-streptomycin, 200 mM L-glutamine, 12.5 mM Hepes buffer 0.05% sodium bicarbonate (Microbiological Associates, Walkersville, MD) and 12% heat-inactivated FCS.

Results:

A. Functional Effects of Depletion of OKT10 Reactive Cells Within Fresh T Cell Subsets:

Experiments were undertaken to examine the functional heterogeneity of the $T4^+$ and $T8^+$ subsets by utilizing OKT10. Initial studies attempted to evaluate the effects of depletion of OKT10 reactive cells in fresh peripheral lymphocytes. PBL were isolated and fractionated into $T4^+$ and $T8^+$ subsets. Each subset was then divided into two populations, one of which was depleted of OKT10 reactive cells by antibody and complement. The $T4^+$ and $T4^+10^-$ cells were then assayed for the ability to enhance Ig production of PWM stimulated autologous B cells. As shown in Table IA depletion of OKT10 reactive cells have no effect on helper function in the $T4^+$ cell subset. Similarly, the $T8^+$ cells were isolated and split into two populations, one of which was depleted of OKT10 reactive cells. The $T8^+$ and $T8^+10^-$ cells were then assayed for their ability to suppress $T4^+$ mediated enhancement of B cell immunoglobulin production. As shown in Table IB, depletion of OKT10 reactive cells within the $T8^+$ subset had no effect on the ability of these cells to suppress Ig production. Thus, depletion of OKT10 reactive cells had no effect on the assayable immunoregulatory functions of fresh $T4^+$ or $T8^+$ T cell subsets.

In Table II, the surface phenotypes of E^+ cells used in these experiments are shown. Freshly isolated T cells contained 5% OKT10 reactive cells. After antibody and complement treatment with OKT10A, the OKT10 reactive cells were totally depleted (as shown by a reactivity less then the control ascites' non-specific

reactivity). Of interest is the change in surface phenotype after the cells have been cultured with PWM for 5 days. As noted before, unselected E^+ cells activated by mitogen show a greater number of cells reactive with OKT10. Those same fresh E^+ cells which have been depleted of their OKT10 reactive cells prior to culture with PWM also showed a greater percent reactivity with OKT10. Figure 2 shows an example of the cytofluorograph display of the OKT10 reactive cells, as compared to the control ascites display. Thus, the activation of fresh T cells by PWM was shown to increase their OKT10 reactivity, and this increased reactivity was unaffected by prior depletion of OKT10 reactive cells from fresh T cells.

B. Functional Effects of OKT10 Depletion on Activated Cells

To further investigate the functional heterogeneity of activated T cells, a series of studies were undertaken to see what effects depletion of OKT10 reactive T cells after activation would have on T cell regulation of B cell antibody production and on T cell mediated cytotoxicity.

1. Help and Suppressive Functions Within the $T4^+$ Activated Subsets are Unaffected by Depletion of OKT10 T Cells:

In the following experiment, the effect of removal of OKT10 reactive cells within the activated $T4^+$ population was studied. E^+ cells were activated with PWM for 4 days. Study of the cell surface of these cells revealed that the % reactivity with OKT10 increased from 5% to 30% of the $T4^+$ cells after activation. The cells were harvested and then fractionated into $T4^+$ and $T4^+10^-$ subsets with antibody and complement treatment. Graded

numbers of either $T4^+$ or $T4^+10^-$ cells from the first culture were then added to a secondary culture containing PWM-driven autologous B cells. PFC activity was measured 5 days later and are shown in Figure 3A.

As previously shown (8,13) at low ratios of T cells to B cells, $T4^+$ cells increased B cell differentiation as measured by PFC. At high T cell to B cell ratios, addition of $T4^+$ cells reduced the PFC response. The $T4^+$ cells depleted of OKT10 reactive cells showed the same effect on B cell differentiation.

In a parallel experiment, T cells were activated by PWM, and then fractionated into $T4^+$ and $T4^+10^-$ subpopulations. These cells were then irradiated and graded numbers placed in a secondary culture containing PWM-driven autologous B cells. PFC activity after 5 days was assayed and shown in Figure 3B. As previously reported (8,13), irradiated $T4^+$ cells also induced B cell differentiation, but only at high ratios of T cells to B cells. This enhancement of PFC response by irradiated $T4^+$ cells was unaffected by depletion of OKT10 reactive cells within the $T4^+$ population. Thus, depletion of OKT10 reactive cells from activated irradiated or non-irradiated $T4^+$ cells did not affect their ability to regulate B cell immunoglobulin production.

2. <u>OKT10 Reactive Cells Responsible for Effector Phase of T Cell Mediated Cytotoxicity</u>:

The next set of experiments investigated the effect that depletion of OKT10 reactive cells had on T cell mediated cytotoxicity. E^+ cells were sensitized by allogeneic E^- irradiated cells. After 6 days, the cells were harvested and divided into two

populations. One population was incubated with OKT10A and treated with complement thus depleting the $OKT10A^+$ cells. The other population was treated with complement alone. Both populations were then assayed for cytotoxic activity against allogeneic targets. As shown in figure 4B, the E^+ cells depleted of $OKT10^+$ cells do not mount a substantial cytotoxic response. In contrast, the E^+ cells treated with complement alone do develop CTL activity.

Prior to stimulation, the $OKT10^+$ population in the E^+ cells was 5%. In the activated E^+ population the percentage of cells that reacted with OKT10 rose to 23%. After treatment, with OKT10A and C', almost all the $OKT10^+$ cells were eliminated.

In previous studies it has been shown that the precursors of T cell cytotoxicity are contained in the $OKT8^+$ population. (14). Experiments were undertaken to determine if this precursor population was also reactive with OKT10A. E^+ cells were isolated from PBL and split into two populations. One population was treated with complement alone. Another population was depleted of $OKT10^+$ T cells with antibody and complement. Both populations were stimulated by mixed lymphocyte reaction for six days in culture and then assayed for cytotoxic response.

In Figure 4A, the cytotoxic activity of the two populations is shown. The population depleted of $OKT10^+$ cells before stimulation has the same level of response as the control E^+ population treated with complement alone. Prior to the stimulation, treatment of E^+ cells with antibody and complement successfully depleted the respective T cell subset. After the six day mixed alloantigen

activation the phenotypic profile of the E^+ cells change. In both populations there is an increase in the number of cells that react with OKT10. The reactivity of these cells with OKT3, OKT4, and OKT8 does not change drastically.

3. $T8^+$ Suppression Unaffected by Depletion of OKT10 Reactive Cells:

In the following experiments the effect of removal of OKT10 reactive cells on the suppressor function of $T8^+$ cells was tested. E^+ cells were isolated and activated with PWM for 3-5 days. The cells were harvested and then $T8^+$ cells were isolated by treatment with OKT4 and complement. These cells were split into two populations, one of which was depleted of OKT10 reactive cells using antibody and complement. These two populations $T8^+$ and $T8^+10^-$ were then tested for their ability to suppress $T4^+$ induced B cell differentiation. Graded numbers of $T8^+$ and $T8^+10^-$ T cells were added to a fixed mixture of B cells and $T4^+$ helper cells in the presence of PWM. After 5 days, cultures were assayed for plaque-forming cells (PFC) activity.

In Table III, the PFC responses of these populations are graphed as the % change (suppression) of B cell PFC response. In repeat experiments, $T8^+$ cells were able to decrease $T4^+$ induced PFC response. The $T8^+$ cells depleted of OKT10 reactive cells ($T8^+10^-$) also were able to decrease the number of PFC's formed. Thus, depletion of OKT10 reactive cells did not affect the suppressor function of non-irradiated $T8^+$ cells.

The surface phenotypes of these cells are shown in Table IV. E^+ cells activated by PWM were fractionated into $T8^+$ and $T8^+10^-$ subsets, then again activated by PWM for 5 days and

reassessed for surface phenotypes. Due to the activated state of the cells non-specific binding of fluorescein-conjugated $(Fab)_2$ goat anti-mouse IgG is high, as indicated by % reactivity with control ascitic fluid. Still, there is a significant subset of cells reactive with OKT10 in the $T8^+$ population (approximately 20% over control), while the cells depleted with OKT10 and C show no reactivity above ascitic control. Interestingly, after reactivation with PWM, the $T8^+$ cells again have a large OKT10 reactive population while the cells depleted of OKT10 reactive cells after the first PWM activation show no reactivity above ascitic control. Thus depletion of the OKT10 reactive cells from inactivated $T8^+$ population eliminated the ability of the T cells to generate OKT10 reactive cells.

4. Irradiated Activated $T8^+$ Cells Depleted of OKT10 Reactive Cells Enhance B Cell Differentiation:

Recent studies (3) have revealed that at some levels of helper activity mediated by fresh $T4^+$ cells, irradiated activated $T8^+$ cells amplify PFC response. To determine the effect of depletion of OKT10 reactive cells on this activity, E^+ cells were activated for 6 days by PWM. Cells were then fractionated into $T8^+$ and $T8^+10^-$ populations by antibody and complement. Each of these populations was then divided into two fractions, one of which was irradiated. Graded numbers of these four populations of T cells were then added to a mixture of B cells and fresh $T4^+$ cells and cultured for 6 days, at which time PFC response was measured. As shown in Figure 5A, and described earlier in this paper,

non-irradiated, activated $T8^+$ cells suppress B cell differentiation even after depletion of OKT10 reactive cells.

Figure 5B shows the results of the experiments involving the irradiated test populations. The assay culture containing $T4^+$ cells and B cells alone produced a high level of immunoglobulin production. At the level of help generated in this experiment activated, irradiated $T8^+$ cells suppressed B cell differentiation. In contrast after depletion of the OKT10 reactive cells, $T8^+10^-$ activated irradiated cells were unable to suppress a $T4^+$ induced B cell differentiation at low T to B cell ratios. At high T to B cell ratios, the $T8^+10^-$ cells resulted in a dramatic enhancement in the number of immunoglobulin secreting cells. Thus, at the level of $T4^+$ induced B cell differentiation examined, activated irradiated $T8^+$ cells depleted of OKT10 reactive cells did not suppress PFC response and enhanced this response at high T to B cell ratios.

Previous results have demonstrated that irradiated activated $T8^+$ cells do not function as direct inducers of B cell differentiation, but instead require the presence of $T4^+$ fresh cells to mediate this effect. Experiments in which B cells were cultured in the presence of PWM with $T8^+$ or $T8^+10^-$ activated irradiated cells without fresh $T4^+$ cells showed no increased PFC activity over that of a control culture containing B cells with PWM alone. Thus, the enhancing properties of activated, irradiated $T8^+10^-$ cells were dependent on the presence of fresh $T4^+$ cells.

C. Functional Effects of OKT10 Antibody in the Absence Of Complement:

The possible functional role of the OKT10 cell surface molecule was studied by placing the antibody in functional assays in the absence of complement.

1. Lack of Inhibition of CML by OKT10 or OKT10A in The Absence of Complement:

In the following experiment the effect of OKT10 and OKT10A antibodies at the effector phase of T cell mediated cytotoxicity was examined. E^+ cells were sensitized in vitro to irradiated allogeneic E^- cells for 6 days after which the cells were assayed for CML activity in the presence of OKT3, OKT10, OKT10A, and control ascitic fluid. Results are shown in Figure 6. As previously reported (16), the OKT3 antibody blocked specific killing in the CML assay as measured by ^{51}Cr release. In contrast, the specific killing of cytotoxic T cells in the presence of OKT10 or control ascites is identical. The specific cytotoxicity in the presence of OKT10A was identical to the control at a 40:1 ratio, though at lesser ratios the amount of CML was slightly higher in the presence of OKT10A. Thus, neither OKT10 nor OKT10A, in the absence of complement could block CML activity.

2. Lack of Effect of OKT10 on In Vitro Proliferative Studies

To assess whether OKT10 could affect alloantigen-triggered T cell proliferation E^+ cells were sensitized in vitro to irradiated allogeneic E^- cells in the presence of antibody for 3 and 6 days, and assayed for proliferation by ^3H-thymidine incorporation. As shown in Table V, the proliferation in the presence of OKT10 was similar to that in presence of OKT8 and B_1 antibodies and to the

media control, both at Day 3 and Day 6 of in vitro sensitization. Thus, OKT10, in the absence of complement, did not prevent proliferation to alloantigen.

Discussion

In the above experiments, a pair of monoclonal antibodies, OKT10 and OKT10A, were utilized to further define functional heterogeneity within $T4^+$ and $T8^+$ T cell subsets. Depletion of OKT10 reactive cells with antibody and complement treatment eliminated the cytotoxicity response of activated T cells to an allogeneic target. Similar depletion of OKT10 reactive T cells within the $T8^+$ activated non-irradiated population failed to affect the suppression of $T4^+$ induced B cell differentiation. In contrast, the depletion of OKT10 reactive cells from the $T8^+$ activated irradiated population converted their suppressive effect on $T4^+$ function to an enhancing effect of $T4^+$ induced B cell Ig production. Taken together, this data illustrates that the multiple functions of the $T8^+$ population of T cells are most likely performed by different subpopulations of these cells which are defined by their reactivity with the OKT10 antibody.

In the cytotoxicity experiments, OKT10 depleted E^+ populations were shown to be able to generate a cytotoxic response after six days of stimulation in a mixed lymphocyte reaction. Such depletion of the OKT10 reactive population after stimulation eliminated the cytotoxic effector cells within the T cell population. Study of cell surface phenotypes indicate these effectors of CML attain the OKT10 reactive molecule during allogeneic activation. Similar depletion of the $OKT8^+$ T cell subset, either before or after activation, eliminates the cytotoxic response to T cells to allogeneic targets. (36,89).

In previous studies it had been shown that OKT10 antigen appears on the effectors of natural killer cell cytolysis (85). Given evidence of its presence of T cell killers, experiments were undertaken to ascertain if the OKT10 molecule itself was necessary for either generation of killer cells or for the killing function itself. The approach utilized was to attempt to block function by placing the antibody in culture in the absence of complement. Experimental results showed OKT10 was unable to block proliferation to alloantigen as measured by ^{3}H-thymidine incorporation after 5 days of stimulation. Similarly, neither OKT10 nor OKT10A, (antibodies specific for different epitopes of the OKT10 reactive molecule) had a blocking effect on cytotoxic function. In contrast, the OKT3 molecule which in previous studies was able to block cytotoxic function at the effector phase (60) did decrease the killing seen in this experiment.

Drawing conclusions from experiments attempting to block T cell function by placing antibodies in culture in the absence of complement is difficult for several reasons. As previous studies examining antibodies reactive with the OKT4 and OKT8 antigens have shown (37,64,65), there can be great variation in the effects that monoclonal antibodies specific for different epitopes of antigens, can exert on T cell function. Another difficulty studying the OKT10 antigen is the low level of its expression on the cell surface as observed on cytofluoragraph examination. Thus, the concentration of antibody used in blocking experiments might not have been sufficient to affect a significant number of cells. Therefore, though it is tempting to conclude that the OKT10

molecule is not necessary for either proliferation to alloantigen or cytotoxic function, further study, with antibodies to other epitopes of the OKT10 reactive molecule, could better define this conclusion.

Another set of experiments studied what function OKT10 reactive cells in the peripheral blood serve during T cell-driven B cell differentiation. Depletion of OKT10 reactive cells from fresh peripheral T cells had no effect on either $T4^+$ helper functions or $T8^+$ suppressive function. This was not surprising in light of the small percentage of fresh peripheral T cells reactive with OKT10. In addition, we observed that after depletion of OKT10 reactive cells from peripheral T cells and reactivation in our assay system, there was a reemergence of OKT10 reactive cells. Thus, if the presence of the OKT10 molecule on the cell surface is a marker for a functionally defined T cell population, depletion of OKT10 reactive cells from fresh T cells and assay in a system utilizing PWM activation in the presence of B cells, does not offer an optimal experimental approach.

Experiments were then directed at T cells polyclonally activated by PWM, which by phenotypic studies showed an increase in the percentage of cells reactive with OKT10 in both the $T4^+$ and $T8^+$ populations.

Previous studies with activated, non-irradiated $T4^+$ cells have shown that addition of graded numbers o. these cells to B cells resulted in a leveling off of the enhancement in PFC response at a relatively low $T4^+$ cell concentration, and a subsequent decline in PFC response with higher concentrations of $T4^+$ cells (13). This

has been attributed to a regulatory loop within the $T4^+$ population involving both suppressor and helper cells which help modulate immunoglobulin production. (13).

In the experiments presented in this paper, depletion of OKT10 reactive cells within the $T4^+$ activated non-irradiated T cells subset had no effect on the characteristic immunoregulation of B cell differentiation stated above. Nor, did depletion of OKT10 reactive cells have an effect on the radioresistant helper function mediated by activated, irradiated $T4^+$ cells.

Several experiments were performed studying the effect that depletion of OKT10 reactive cells had on suppressor function generated by non-irradiated activated $OKT8^+$ cells. In none of these experiments was the suppression of $T4^+$ induced B cell differentiation affected by depletion of OKT10 reactive cells. Though it is tempting to conclude that OKT10 reactive cells within the $T8^+$ subset do not exert suppressor function, there are several problems inherent in a negative selection assay that must be addressed.

The major concern with analyzing these results concerned whether the cell population labelled $T8^+10^-$ were actually without cells reactive with the OKT10 antibody. This was of even greater concern given the above results of the antibody and complement studies with fresh T cells which revealed that the emergence of OKT10 reactivity with PWM activation was not a clonal expansion of OKT10 reactive cells that could be eliminated by depletion of reactive cells prior to activation. This was supported by the studies of Goldstein involving the pertubation of T cells which

revealed that the OKT10 molecule was probably present in an unexpressed form in resting T cells. (87).

To investigate this problem, activated $T8^+$ cells were depleted of their OKT10 reactive cells and then recultured with PWM. Studies of cell surface phenotype after this second culture revealed that there had been no reemergence of OKT10 reactive cells after this depletion. This result is stated with some reservation due to the technical difficulty in phenotyping activated T cells. This is related to the emergence of a great deal of non-specific binding of the Fc portion of antibodies in these activated T cells as indicated by the 50% reactivity of the control ascitic fluid. In trying to control for this binding the cursor (threshold for the amount of fluorescein binding necessary to be assessed as positive reactivity) was raised. This accounts for the lowered reactivity of these $T8^+$ cells with OKT3 and OKT8, revealed in Table IV, which on inspection of binding patterns on the cytofluoragraph revealed that all the cells were binding some antibody, but those cells expressing low quantities of these antigens were now being recorded as negative reacting cells. This same technical aberration could cause some OKT10 reactive cells expressing low quantities of the antigen to be identified as non-reactive cells.

A second difficulty in analyzing these experiments is that which is inherent to a negative result in a depletion study. If the amount of a function generated by a given cell population is in excess of that which an assay could measure, then depletion of a subset of these cells would not necessarily result in a measurable change in that function. Thus, even though activated $T8^+10^-$ cells

still are able to suppress $T4^+$ induced B cell differentiation, it is not necessarily true that the depleted OKT10 reactive cells do not have any suppressive activity. To try to minimize this problem, the studies were done using graded numbers of T cells in order to increase sensitivity to any changes in function caused by the depletion of the OKT10 reactive cells.

Another approach to this problem is to select cells with OKT10 reactivity and use these "positive selected" cells in functional assays. This was attempted several times utilizing the rosetting technique of Strelkauskas (90), without success. These attempts were made with a goat antibody to mouse IgG, prior to the discovery that OKT10A was an IgM antibody.

Recent studies by Thomas, et al, have demonstrated activation of the $OKT8^+$ subset results in the emergence of T cells with counter-balancing immunoregulatory properties (3). Activated irradiated $T8^+$ cells suppress the generation of PFC only when the level of $T4^+$ helper activity was optimal. However, when the level of help was either sub or supra-optimal these same activated irradiated $T8^+$ cells amplified the PFC response. This amplification function of the $T8^+$ cells was strictly dependent on the presence of fresh $T4^+$ cells. The activated irradiated $T8^+$ cells added to B cells alone had no effect on PFC response.

In analyzing these results the authors offer three potential explanations:

1. $T8^+$ and $T4^+$ cell populations rather than having distinct activities possess overlapping functions.

Evidence for this comes from recent studies by Schlossman's group, and others, (58, 91,92,93), that $T4^+$ and $T8^+$ subsets may have distinct functions depending on cell-surface receptors for Class I or Class II HLA antigens. They have shown that $T4^+$ cell clones which recognize Class II antigens can function as cytotoxic cells specific for Class II specificity, as well as helper function. (93,94). Analogous findings were seen with $T8^+$ cytotoxic clones which had Class I HLA specificity. (91,92).

2. A second model for these findings states that precursors of functionally opposing subsets exists within both the $T4^+$ and $T8^+$ cell population. The authors draw analogies to the murine system where the Ly1,2,3 cell can be driven to differentiate along either helper or suppressor pathways. (19).

3. The third possible explanation of the data infers that irradiated $T8^+$ cells do not amplify B cell differentiation themselves, but are inducers of contrasuppression, an immunoregulatory phenomenon previously described in the murine system by Gershon. (49). The authors point out that the inducer of contrasuppression in the mouse is an Ly2 cell and the effector cell of this function bears the Ly1 phenotype. Thus, the human findings of a $T8^+$ cell being the inducer cell for this function and the $T4^+$ fresh cell the effector of contrasuppression would seem analogous.

In an accompanying editorial, D. Green presents other evidence for contrasuppression in the human system. (97). Green further attempts to define the immunoregulatory effects seen in Thomas' experiments by proposing that an amplifier of suppression (a/k/a "level 2 suppression") which has been postulated in the murine system and also in the human system (95,96) might be responsible for the results. Thus, a radiosensitive $T8^+$ cell which amplifies suppressive function may override the contrasuppressor effect of another $T8^+$ cell.

Utilizing the OKT10 antibody, experiments reported above focused on the immunoregulatory functions of activated $T8^+$ cells. Both $T8^+$ cells and $T8^+$ cells depleted of OKT10 reactive cells ($T8^+10^-$), non-irradiated, suppressed B cells differentiation, but, at the level of $T4^+$ induced B cell differentiation examined, the irradiated $T8^+$ cells still showed suppressive activity while the $T8^+10^-$ cells amplified B cell function. These effects were completely dependent on the presence of $T4^+$ fresh cells.

These results are compatible with all three models of $T8^+$ enhancing function proposed by Thomas, et al. The first model proposed that irradiation of activated $T8^+$ cells eliminates a population of radiosensitive suppressor cells which allows the enhancing effect of another subset of $T8^+$ cells to be expressed at sub or supra optimal levels of help, but not at optimal levels. The depletion of OKT10 reactive cells could be eliminating a population of radioresistant suppressor cells which then allows enhancing activity to be expressed at the optimal level of help, at which this experiment took place.

The second model proposes that there is a precursor cell within the $T8^+$ cell population capable of differentiating into either a suppressor or helper cell. The depletion of OKT10 reactive cells could have either eliminated a subset of suppressor cells allowing enhancing activity to be expressed or it could have eliminated the precursor cell thus eliminating the generation of suppressor cells needed to regulate an already present $T8^+$ population with enhancing properties.

The third model proposes that there are $T8^+$ cells capable of inducing contrasuppression activity of $T4^+$ cells, and a subset of $T8^+$ cells that can amplify suppressor activity. Thus, depletion of OKT10 reactive cells could have eliminated the cells capable of amplifying suppressor activity, allowing the contrasuppression function to be expressed.

Irrespective of which model is correct, the experiments in this study indicate that depletion of OKT10 reactive cells within the $T8^+$ activated irradiated T cell subset can change the effect of these cells on $T4^+$ induced B cell differentiation from one of suppression to one of enhancement.

Table VI lists different T cell subsets and the effect of depletion of OKT10 reactive cells from within these subsets on their immune function. These findings can be useful in several different modalities. Foremost, the antibody will be able to aid in the investigation of T cell immune function and will help to define the heterogeneity of T cell subsets.

Clinically, these findings can help define the pathophysiology of several immune related diseases. For instance, studies of

infectious mononucleosis have found peripheral T cells in these patients have a high percent reactivity with OKT10, which these authors proposed was due to an increased level of immature T cells. (88). The finding that these cells are responsible for cytotoxic function and possibly inducement of suppression implicate different mechanisms of immune response to EBV infection of human B cells. Thus, T cells may be attempting to kill infected cells or in contrast, may be attempting to immunoregulate B cell Ig production. Hopefully, further definition of the role of OKT10 reactive cells can come with experiments either utilizing T cell cloning technology or improved techniques in positive selection.

Abbreviations

C	-	complement
CML	-	cell mediated lympholysis
Con A	-	concanavlin A
CTL	-	cytotoxic T lymphocyte
EDTA	-	ethylenediaminetetraacetic acid
FCS	-	fetal calf serum
HEPES	-	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
Ig	-	immunoglobulin
MHC	-	major histocompatibility complex
MLC	-	mixed lymphocyte culture
MLR	-	mixed lymphocyte (leukocyte) reaction
PBL	-	peripheral blood lymphocyte
PFC	-	plaque forming colony (cells)
PHA	-	phytohemagglutin
PWM	-	pokeweed mitogen
SRBC	-	sheep red blood cells

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ADDENDUM

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Table I: Functional Effects of Depletion of OKT10 Reactive Cells from Within Fresh T Cell Subsets.

A. Helper Function:

Number of T Cells Added per culture*	$T4^+$ Cells Added PFC/10^6 Cells	$T4^+10^-$ Cells Added PFC/10^6 Cells
0	1,000	1,000
0.05×10^6	18,000	17,680
0.10×10^6	21,920	27,360
0.20×10^6	16,800	19,040
0.50×10^6	12,560	16,920
1.00×10^6	10,300	8,140

B. Suppressor Function

Number of T Cells Added per assay culture¢	$T8^+$ Cells Added		$T8^+10^-$ Cells Added	
	PFC/10^6 Cells	%Suppression#	PFC/10^6 Cells	%Suppression#
0	11,990	-	11,990	-
0.20×10^6	9,790	18	7,050	41
0.50×10^6	2,670	77	3,780	68
1.00×10^6	2,010	83	2,300	81

* Graded numbers of T Cells added to standard culture of 1×10^6 B cells and 10 ug PWM

¢ Graded numbers of T cells added to standard culture of 1×10^6 B cells, 0.05×10^6 $OKT4^+$ T Cells, and 10 ug PWM.

$$\# \%\text{Suppression} = \left[1 - \frac{\text{PFC (Experimental Culture)}}{\text{PFC (Standard Culture)}} \right] \times 100$$

Table II: Cell Surface Phenotypes of Isolated T Cell Subsets Before and After PWM Activation:

	Surface Phenotype % Reactivity with Monoclonal OKT Antibody				
	Ascites	OKT3	OKT4	OKT8	OKT10
Fresh E^+ Cells					
Ascites and C (E^+)	1	88	44	42	5
OKT10A and C (E^+10^-)	3	88	44	40	1
After 5 days PWM Activation (first culture)					
Ascites and C (E^+)	9	93	66	47	27
OKT10A and C (E^+10^-)	7	95	61	11	36

Table III: Depletion of OKT10 Reactive Cells Does Not Affect $T8^+$ Suppressor Functions.

Number of Activated T Cells (first culture) added to a Secondary Culture*	$T8^+$ Cells Added		$T8^+10^-$ Cells Added	
	PFC/10^6 Cells	%Suppression@	PFC/10^6 Cells	%Suppression@
Expt. 1				
0	27,000±700	-	27,000±700	-
$0.1x10^6$	6,720±180	75	4,840±560	82
$0.2x10^6$	11,360±680	58	2,840±310	89
$0.5x10^6$	4,520±800	83	1,640±140	94
$1.0x10^6$	4,040±140	85	8,640±560	68
Expt. 2				
0	33,340±6924	-	33,340±6924	-
$0.1x10^6$	3,680±540	89	17,740±2300	46
$0.2x10^6$	760±±00	98	13,280±1720	60
$0.5x10^6$	640±40	98	5,540±200	83
$1.0x10^6$	840±80	98	4,140±200	87
Expt. 3				
0	2,269±39	-	2,269±39	-
$0.2x10^6$	1,760±37	22	1,911±65	18
$0.5x10^6$	963±62	58	1,018-	55
$1.0x10^6$	295-	87	330-	85

* Graded numbers of activated T Cells (first culture) were added to standard cultures containing $1.0x10^6$ B Cells, $0.05x10^6$ fresh $T4^+$ cells, and 10 ug PWM

@ % Suppression = $\left[1 - \frac{\text{PFC (Experimental Culture)}}{\text{PFC (Standard Culture)}} \right] \times 100$

Table IV: Cell Surface Phenotypes of Activated T Cells after Initial Isolation and after Reactivation with PWM:

	Surface Phenotype % Reactivity with Monoclonal OKT Antibody				
	Ascites	OKT3	OKT4	OKT8	OKT10
T Cells After 5 day PWM Activation:					
Treated w/OKT4 & C ($T8^+$)	16	78	16	46	35
Treated w/OKT4,OKT10 & C ($T8^+10^-$)	36	72	35	63	36
T Cells After 5 days Reactivation:					
($T8^+$)	50	80	50	78	74
($T8^+10^-$)	50	80	50	68	51

Table V: Lack of Inhibition of Alloantigen - Triggered T Cell Proliferation by OKT10 Antibody.

Monoclonal Antibody	Day 3 MLR*	Day 6 MLR*
Media Control	38,000±2300	64,700±510
OKT10	42,800±1800	79,000±880
OKT8	-	63,000±840
OKB_1	-	80,000±4500

* Results are expressed as counts of $[3_H]$-thymidine incorporation ± SEM

Table VI: Summary of T Cell Subsets Isolated With OKT4, OKT8 and OKT10 Antibodies.

Isolated Subset	Functions: Help (Enhancement) of B Cell Differentiation	Functions: Suppression of B Cell Differentiation	Functions: Cytotoxicity
Fresh Cells			
$T4^+$	+	-	
$T4^+10^-$	+	-	
$T8^+$	-	+	
$T8^+10^-$	-	+	
Activated Cells			
$T4^+$	+	+	
$T4^+10^-$	+	+	
$T4^+$ irradiated	+	-	
$T4^+10^-$ irradiated	+	-	
$T8^+$	-	+	+
$T8^+10^-$	-	+	-
$T8^+$ irradiated	-*	+*	
$T8^+10^-$ irradiated	+*	-*	

* At optimal $T4^+$ induced B Cell IG production

\+ Isolated Subset Exhibits Function

\- Isolated Subset Lacks Function

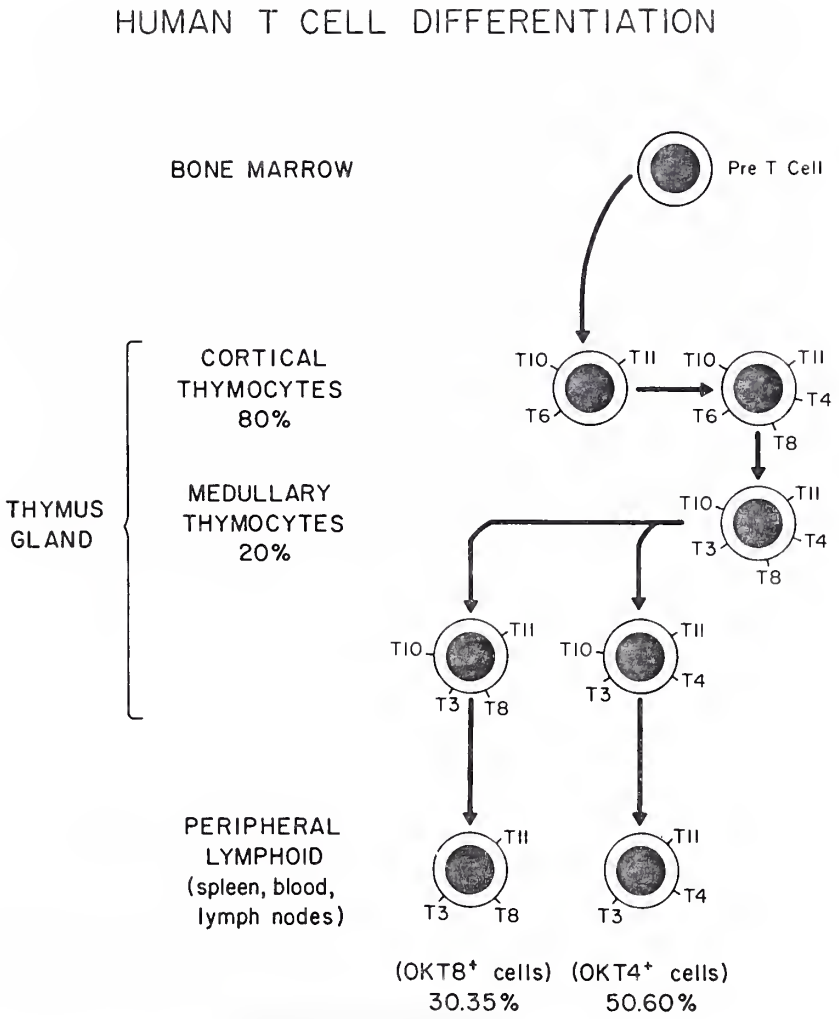

Figure 1: Stages of T-Cell Maturation in Humans.

Several stages of T cell development can be defined on the basis of reactivity with monoclonal antibodies.

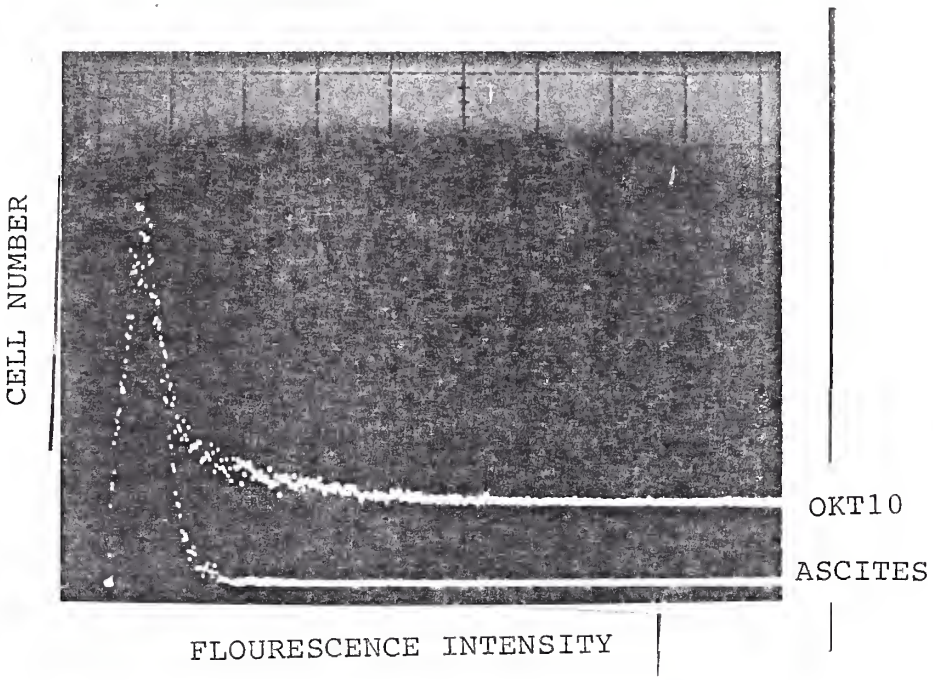

Figure 2: Cytofluoragraph Display of OKT10 Reactivity of Activated T Cells.

Fluorescence histogram of OKT10 on activated (by PWM for 4 days) E^+ cells (top), as compared with control ascitic fluid (bottom). Fluorescence intensity is a result of the number of monoclonal antibodies bound per cell.

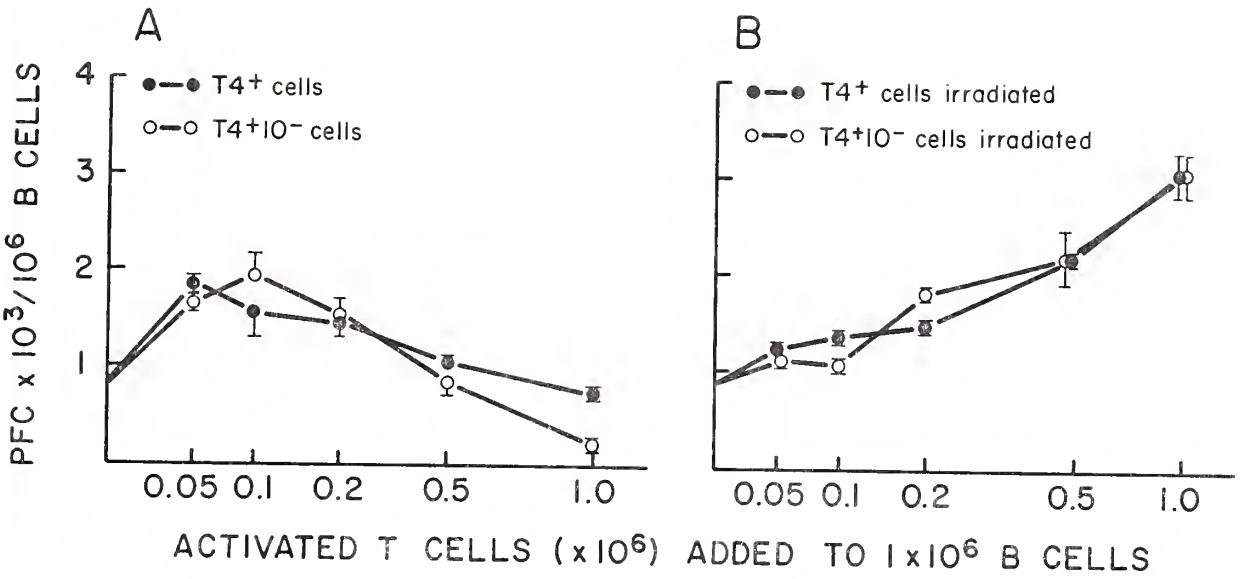

Figure 3: Depletion of OKT$_{+}$10 Reactive Cells does not Affect the Function of T4^{+} Activated Cells.

The standard culture contained $1x10^{6}$ B Cells and 10ug of PWM. To this system were added graded numbers of either T4^{+} cells (•-•) or T4^{+}10^{-} cells (o-o) cultured with PWM during 96 hours of previous culture. These cells were either added to system directly (A) or first irradiated with 1250 rads (B): After 5 days cultures were harvested and assayed for PFC activity.

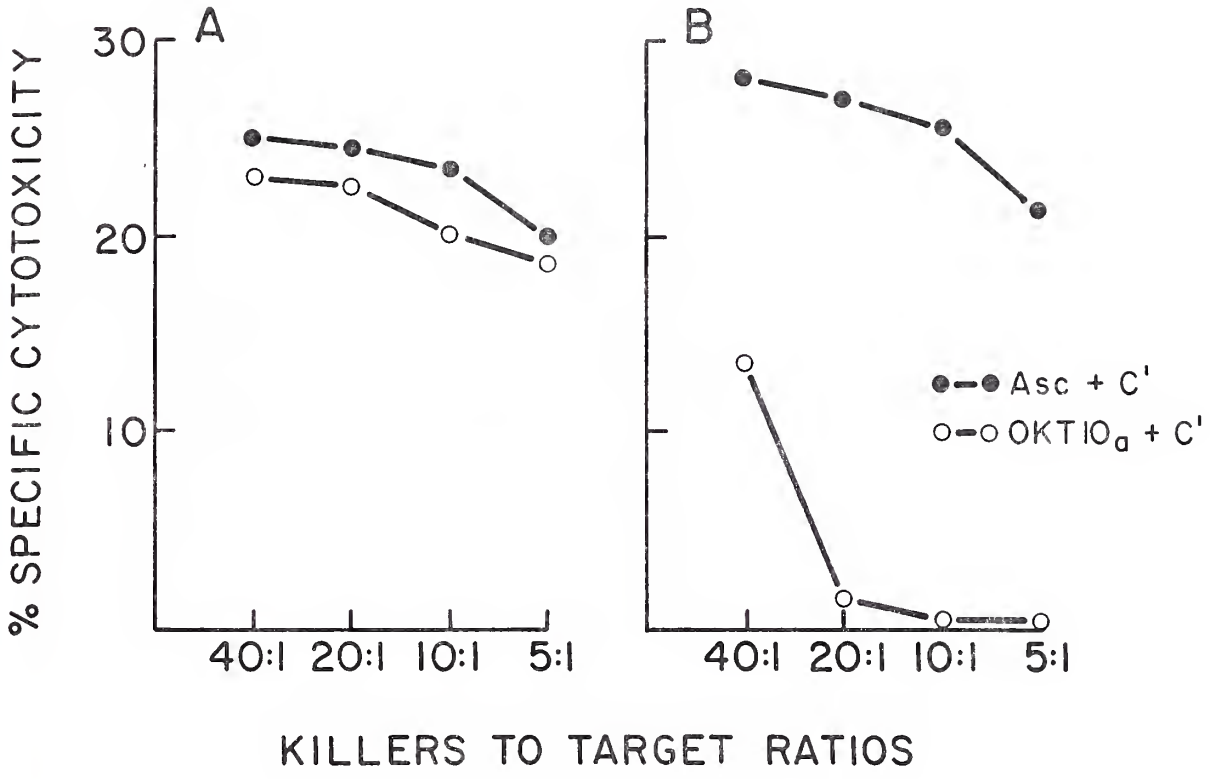

Figure 4: Cytotoxic Effector but not precursor cells react with OKT10. Responder T cells, either before (A) or after (B) in vitro sensitization to irradiated (1250 rad) allogenic stimulators were treated with C alone (●-●) or OKT10A plus C (o-o). Viable cells from each treatment group were assayed at the killer-to-target ratios indicated, in a 5-hour cellular cytotoxicity assay against ^{51}Cr-labelled cells autologous to the stimulators.

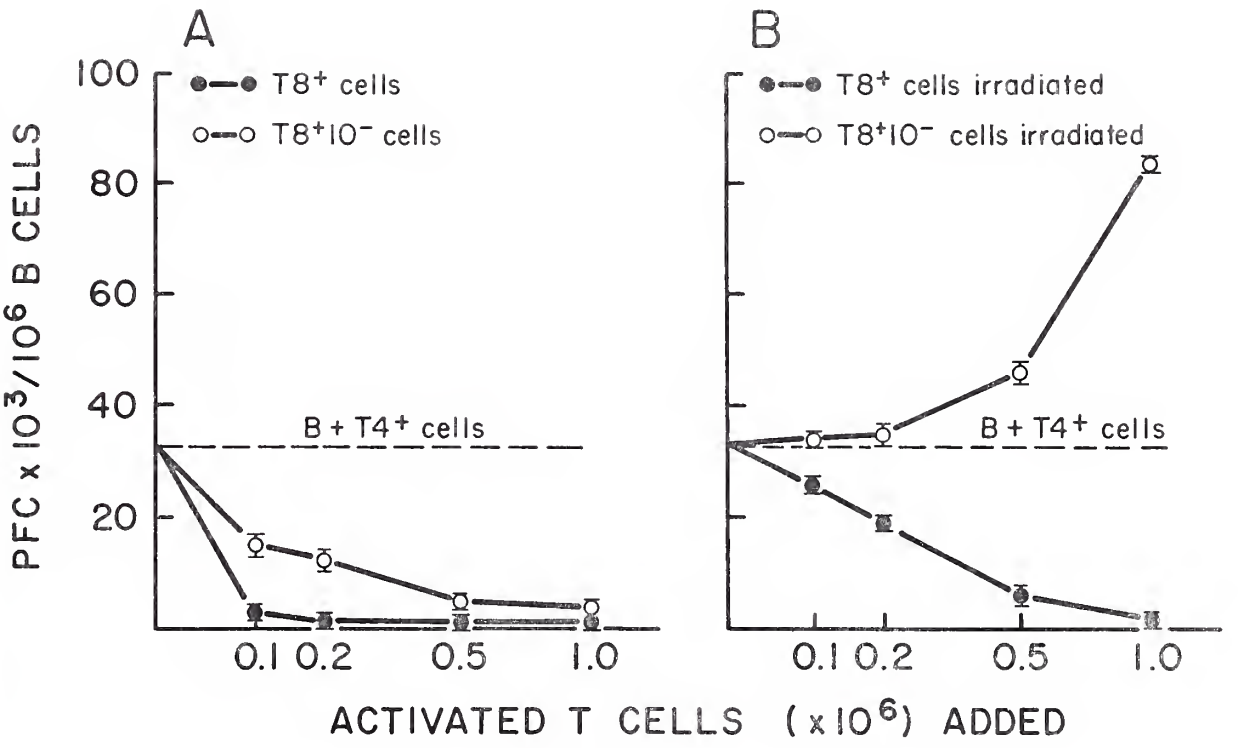

Figure 5: The immunoregulatory effect of $T8^+$ activated irradiated cells on $T4^+$ induced B cell differentiation is converted from suppressive to enhancement with depletion of the OKT10 reactive population from within the $T8^+$ subset.

(A) Graded numbers of non-irradiated $T8^+$ cells (●-●) or $T8^+$ cells depleted of OKT10 reactive cells (o-o) from first culture period were added to standard culture of 0.05×10^6 fresh $T4^+$ cells and 1×10^6 B cells in addition to 10ug PWM.

(B) Graded numbers of $T8^+$ cells (●-●) or $T8^+$ cells depleted of OKT10 reactive cells (o-o) from first culture were irradiated with 1250 rads and added to standard culture of 0.05×10^6 fresh $T4^+$ cells and 1×10^6 B cells in addition to 10ug PWM. In Both (A) and (B), cultures were harvested and assayed for PFC activity 5 days after addition of T cells.

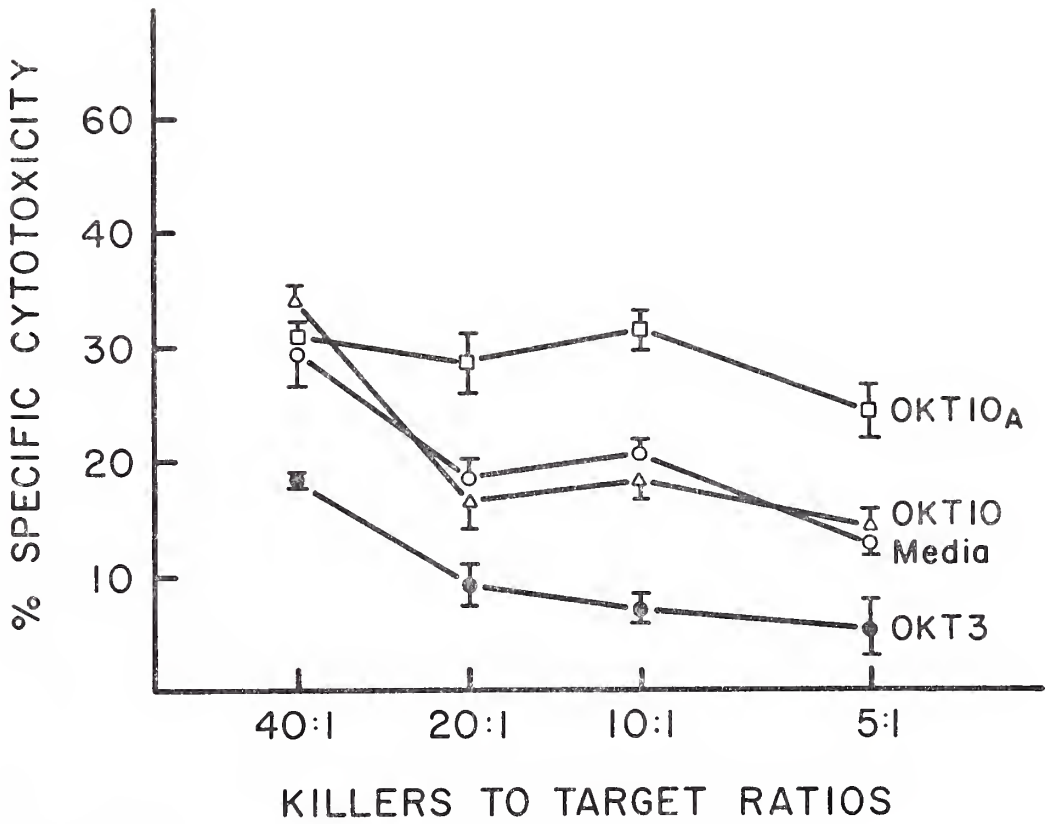

Figure 6: OKT10 and OKT10A Do Not Block T Cell Mediated Cytotoxicity.

Responder T cells were sensitized in vitro to irradiated (1250 rads) allogenic stimulators. Cells were then incubated with either OKT10, OKT10A, OKT3, (1/100) or ascitic control in the absence of complement for one hour, then assayed at the killer to target ratios indicated in a 5 hour cytotoxicity assay against ^{51}Cr labelled cells autologous to the stimulators.

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